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Fluorescent pupal secretion of the papilionid butterfly, *Luehdorfia japonica*

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Abstract Pupae of the papilionid butterfly, *Luehdorfia japonica*, secrete a slightly viscous liquid immediately after pupation from the dorsal surface of the head and thorax, mainly from pro- and meso-thoraces. The secretion is colored pale yellowish-brown and emits greenish-blue fluorescence under light irradiation. The fluid, which gradually covers the dorsal surface of almost the whole body within 2 hours of larval-pupal ecdysis, solidifies along with the melanization and sclerotization of the pupal cuticle. The solidified exudate was almost insoluble in water, but was found to absorb a considerable quantity of water, suggesting that the secretion serves as a moisturizer against desiccation. Although the secretion showed no prominent antimicrobial activity, it seemed to have a weak bacteriostatic effect. Possible ecological roles of the secretion are discussed in regard to the survival strategy of the butterfly.

Key words Pupal secretion, *Luehdorfia japonica*, Papilionidae, fluorescence, chemical defense.

Introduction

Luehdorfia japonica, an Aristolochiaceae-feeding papilionid endemic to Japan, is a univoltine butterfly with a unique life cycle. The butterfly emerges in early spring (in April in many localities of Japan) and larvae grow into pupae in about 2 months (mid June). Pupation usually takes place on dead twigs or stones in a dark and somewhat wet environment close to the ground. Pupae pass summer and autumn in diapausing state, and hibernate as pharate adults until eclosion in the following spring (Hidaka *et al.*, 1971; Ishii and Hidaka, 1982). Pupae have a thick and hard cuticle and this appears to be very important for them to survive hot and cold seasons lasting as long as 10 months. While the cuticle of pupae fresh from ecdysis is very flexible and colored creamy-white, it turns black and tough within about 12 hours due to melanization and sclerotization (Fig. 1A). We have found that shortly after the commencement of larval-pupal ecdysis, pupae begin to exude a liquid colored yellowish-brown from the dorsal integument. As far as we know, no publications have so far referred to pupal secretion, at least in Lepidoptera. We report here on the pupal exudate of *L. japonica* and discuss its possible role in the survival strategy during the long pupal period of the butterfly.

Materials and methods

Insects

Eggs of *L. japonica* were obtained from females collected in Kanagawa and Hiroshima Prefectures, Japan. Larvae fed with fresh leaves of *Heterotropa blumei* or *H. nipponica* (Aristolochiaceae) were reared at 18°C on a 16: 8 hours light: dark photo regime.

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Collection of pupal secretion

Two hours after larval-pupal ecdysis, the secretion was rinsed out of the integument by soaking a pupa in distilled water with continuous swirling for 30 sec. The secretion was collected from a total of 80 pupae using 100 ml of water. The resultant aq. solution of the secretion was filtered to remove miscellaneous insoluble matter, concentrated *in vacuo* below 50°C to *ca* 50 m*l*, and stored in a refrigerator (at 5°C) until use.

Fractionation and chemical analysis of the secretion

An aliquot of the concentrate of the secretion was once lyophilized, and extracted with methanol to afford an insoluble substance (A) and a yellow methanolic solution. The methanol-soluble substances were subjected to thin-layer chromatography (TLC) on silicic acid (Merck TLC plate Silica gel 60) with methanol/ethyl acetate (20/80) as a developer. Spots were detected with a UV lamp.

Fluorescence spectra were measured in methanol with a Hitachi F-2000 spectrofluorometer. IR spectra were recorded on a JASCO IR-810 infrared spectrophotometer. FAB-MS spectra were recorded on a JEOL JMS-DX 303 mass spectrometer using glycerin as a matrix.

Inspection of exudation site

Immediately after larval-pupal ecdysis, the whole body of the pupa was washed with water for several seconds. To make the site of secretion visible, the dorsal surface of the pupa, which was held either horizontally or perpendicularly, was uniformly painted with a commercial white watercolor (Holbein Works, Ltd.). The development of the yellowish-brown color of the exudate was observed 2 hours later.

Moisturizing effect of the secretion

In this experiment, we used pupae of 2 cohorts consisting of 10 individuals each. Immediately after larval-pupal ecdysis, pupae of one group were washed with a large amount of water under ultrasonication and those of the other were left untreated. Approximately 1 day after pupation, treated and intact pupae were transferred into an incubator and kept at 20°C and 90% RH for 24 hours. Sum total of the pupal weight of each group was measured before and after incubation. The total weights of 10 treated and 10 intact pupae at *ca* 50% RH (before incubation) were 5312.9 mg and 5500.3 mg, respectively.

Antimicrobial activity

Since preliminary experiments revealed that the pupal secretion exerted neither fungicidal nor bactericidal activities, its antimicrobial activity was briefly examined. Antifungal activity of the secretion was tested against *Aspergillus niger*, which was precultured at 32°C in a SCD broth medium for 72 hours. The 96-well plates were prepared by dispensing into each well 100 μl of serial dilutions of a test sample (solid material of the secretion), 10 μl of the inoculum, and 90 μl of the nutrient broth or sterilized water. Contents of each well were thoroughly mixed with a pipette and the plates incubated at 32°C for 72 hours. The sample was tested at three doses; 430 μg/m*l*, 215 μg/m*l*, and 108 μg/m*l*. Any growth of the fungus was assessed by microscopically monitoring mycelial growth. Antibacterial activity was tested against two organisms, *Escherichia coli* and *Staphylococcus aureus*. The bacteria were precultured aerobically at 32°C in a SCD broth medium for 24 hours. A mixture consisting of 1 m*l* of a test sample and 10 m*l* of the inoculum was incubated at 32°C. After 72 hours and 7 days, the number of viable cells was counted. The sample was tested at 155 μg/m*l*.

Results and discussion

General observation

The pupal secretion, which gradually covered the dorsal surface of almost the whole body within 2 hours of larval-pupal ecdysis, solidified along with the melanization and sclerotization of the pupal cuticle within about 12 hours. The solidified exudate was almost insoluble in water, and therefore remained on the surface of the pupal cuticle throughout.

Chemical nature of the secretion

Brief examination of the exudate revealed that the secretion contained solid materials at an average of 1.28 mg/individual, and was composed of at least three major substances, *viz.* a methanol-insoluble substance (1) and two fluorescent compounds (2 and 3; 725 μg/individual in total). Compound 2 was detected at Rf 0.3 on TLC, but compound 3 was not developed with the solvent system used. Substance 1 (pale yellow amorphous powder) was considered to be a non-proteineous substance, because a ninhydrin test of its hydrolysis product (10% *aq.* HCl at 80°C for 2 hours) was negative. Although the substance appeared to be

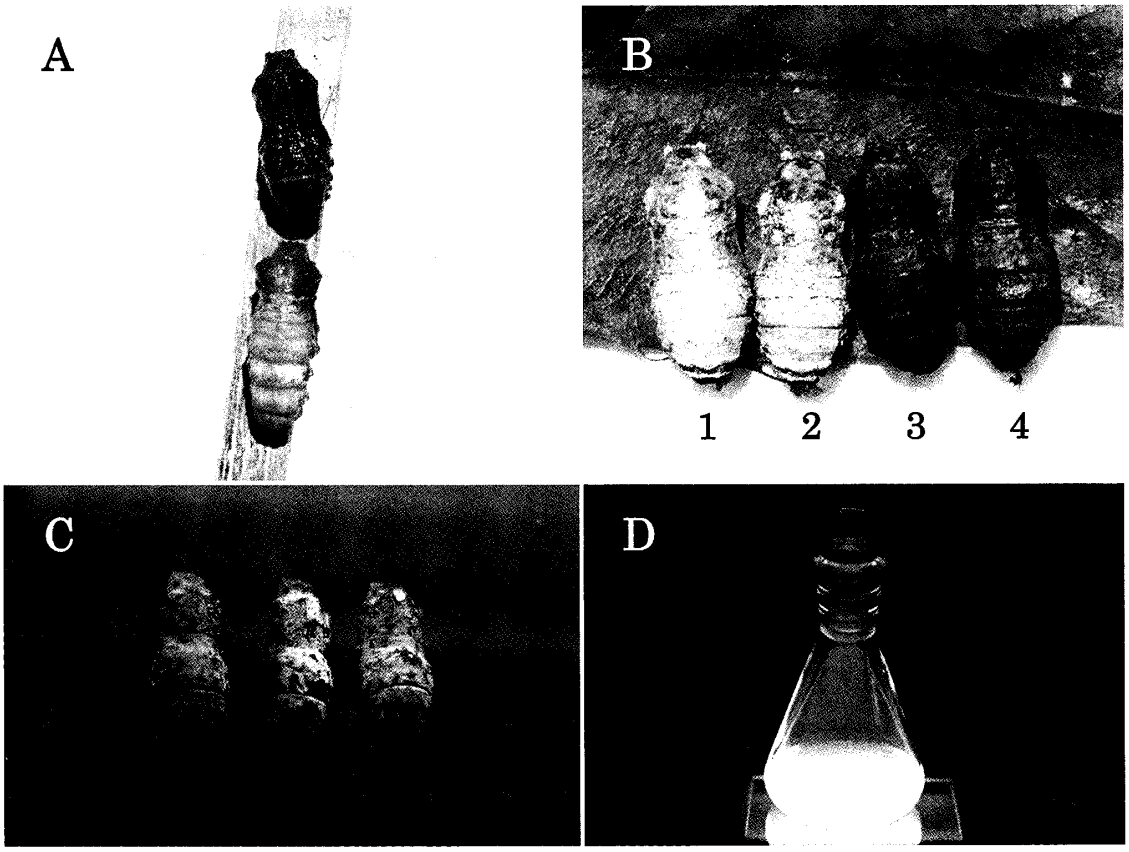

Fig. 1. A: *L. japonica* pupae; upper: 1-day-old pupa, lower: pupa fresh from ecdysis. B1: A pupa of which dorsal surface was painted in white and kept horizontally after larval-pupal ecdysis. A brownish tint due to the exudate can be seen developing only over the upper half (head and thoraces) of the body; B2: A pupa of which dorsal surface was painted in white and kept perpendicularly after ecdysis. Yellowish-brown color is spread as far as the abdomen; B3: A pupa washed with water immediately after ecdysis; B4: An intact pupa. C: Intact pupae under UV irradiation, showing that their dorsal surface is covered with fluorescent substances. D: A methanolic solution of compound 2 emitting greenish-blue fluorescence under UV irradiation.

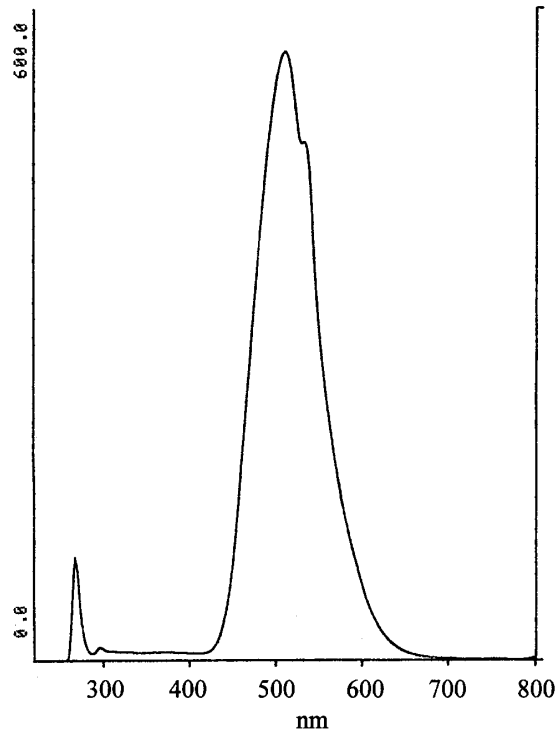

Fig. 2. Fluorescence spectrum of compound 2 in methanol (excited at 265 nm).

contaminated with small amounts of miscellaneous compounds, substance 1 was presumed to be a salt of carboxylated polysaccharide, based on its IR spectra (3276, m; 2937, w; 1599, s; 1390, s; 1118, s; 1048 cm^{-1}, m) and elementary analytical data (C: 37.7%, H: 6.3%, N: 3.3%). Compounds 2 and 3 exhibited similar greenish-blue fluorescence under UV irradiation in methanol (Fig. 1D). The fluorescence spectrum of compound 2 (emission maximum at 512 nm; excitation at 265 nm in methanol) is shown in Fig. 2. Compound 2 exhibited a quasi-molecular ion [$(M+H)^+$] at m/z 265, suggesting a molecular weight of 264. Since purification of compound 3 was not successful, the compound was not pursued further.

The site of exudation

As Fig. 1B clearly shows, the secretion is exuded from the dorsal surface of both the head and the thorax, mainly from the prothorax and mesothorax. No secretion was exuded from the abdomen. We tried to find the outlet of the secretion by means of scanning electron microscopy, but several attempts failed to find any pores or similar architecture on the dorsal surface likely to exude a liquid. Further histological investigations on the epidermal tissue of pupae are needed to locate the secretary organ and clarify the origin of the exudate.

Hygroscopic nature of the secretion

After incubation, the total weight gain of 10 treated (washed) pupae was only 2.6 mg (0.05% of the original weight), whereas that of 10 intact pupae amounted to 48.3 mg (0.88%). This unequivocally implies that the pupal exudate absorbs a significant quantity of water, and thus strongly suggests that the exudate would serve as a moisturizing agent.

Antimicrobial activity

The secretion exerted no fungicidal activity against *A. niger* even at the highest concentra-

Table 1. Tests for antibacterial activity of pupal secretion of *L. japonica*.

Bacteria	Number of cells (per m*l*)		
	0 hours	72 hours	7 days
E. coli	1×10^5	1×10^7	1×10^7
S. aureus	1.4×10^2	5.7×10^3	4×10

tion (430 μg/m*l*). However, mycelial growth was not observed in simple water media. This is suggestive of a weak suppressive effect of the secretion on the proliferation of the fungus. Since an *aq.* solution of the secretion (0.17 w/v% of the solid material) was alkaline (pH: 9.2 at 20°C), the possibility cannot be ruled out that the inhibition of mycelial growth might have been, in part, due to the alkalinity of the secretion. Similarly, no bactericidal activity was found against *E. coli* and *S. aureus* (Table 1). However, the secretion seemed to have a weak bacteriostatic effect against *S. aureus* at the concentration tested. We were not able to measure accurate quantity of the secretion one larva exudes, but *per capita* quantity was estimated at a few tens of micro liters at most. Since one pupa exudes 1.28 mg of secretion (solid material) on average, the actual concentration of the solute on the pupal surface would be much higher than that employed for the antimicrobial assays.

Possible ecological role of pupal secretion

Larvae of *Luehdorfia* species have been reported to exude, from the cervical forked exocrine glands (osmeteria), an odoriferous liquid (Honda, 1980), which has been demonstrated to be biosynthesized *de novo* (Honda, 1900) and to play a defensive role against predatory enemies (Honda, 1983). To the best of our knowledge, however, no publications have hitherto referred to exocrine substances secreted by lepidopterous pupae.

As described above, since *L. japonica* larvae usually pupate in dark and somewhat wet places close to the ground and pupae pass the summer in the diapausing state, and hibernate as pharate adults, the pupae most likely have some defensive maneuvers to protect themselves from considerable climatic changes, microorganisms and predatory enemies. We propose the following defensive roles of the pupal secretion: The secretion may function as (1) a moisturizer that prevents the pupae from desiccation during hot and dry cold seasons, (2) an antimicrobial agent that inhibits the proliferation of fungi and/or bacteria on cuticular surface, and (3) a visual threat (fluorescence emission) to small predatory enemies like lizards or rats. It is feasible that the secretion of pupae in combination with their hard cuticle may provide them with survival benefits during the long immotile stage.

Acknowledgments

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摘　　要

ギフチョウ蛹の蛍光性分泌物 (本田計一・本田　洋・大村　尚)

ギフチョウの蛹は蛹化直後に頭・胸部背面, 主に前・中胸部の背面からやや粘性の液体を分泌する. 分泌物は淡黄褐色を呈し, 特に長波長紫外線照射下で緑青色の蛍光を発する (極大波長: 512 nm). 分泌液は脱皮 (蛹化) 後約2時間でほぼ全身の背面を覆い, 蛹クチクラの着色 (黒化) と硬化の進行に伴って固化する. いったん固化した分泌物は水にほとんど不溶となり, 蛹の期間中ずっと体表面に残る. しかし分泌物は顕著な吸水 (保水) 性を示し, 乾燥に対する保湿剤として機能することが示唆された. 分泌液は弱アリカリ性を示し, 顕著な抗菌性は示さなかったものの弱い静菌作用が認められた. これらのことをふまえて, 本種蛹の生存戦略に関わる分泌物の生態学的な機能について考察した.

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